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Valadão Silva, Daniel; Cabral, Cassia Michelle; Diamantina da Costa, Sarah Stéphane;
de Freitas Souza, Matheus; Alves Ferreira, Evander; Rodrigues Braga, Renan; Mendes
Pereira, Gustavo Antônio; Barbosa dos Santos, José

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Leaf anatomy of cassava (*Manihot esculenta* Crantz. cv. IAC-12) after herbicides application to control weeds in Minas Gerais, Brazil

Anatomia foliar da mandioca (*Manihot esculenta* Crantz. cv. IAC-12) após aplicação de herbicidas para controlar as plantas daninhas em Minas Gerais, Brasil

Daniel Valadão Silva¹, Cassia Michelle Cabral², Sarah Stéphanie Diamantina da Costa², Matheus de Freitas Souza^{3*}, Evander Alves Ferreira², Renan Rodrigues Braga³, Gustavo Antônio Mendes Pereira² and José Barbosa dos Santos²

¹Departamento de produção agrícola. Universidade Federal Rural do Semi-Árido- UFERSA, Brasil. ²Departamento de Agronomia- Universidade Federal dos Vales do Jequitinhonha e Mucuri- UFVJM, Brasil. ³Programa de Manejo Integrado de Plantas Daninhas. Universidade Federal de Viçosa, Brasil. Author for correspondence: freitasouza@yahoo.com.br

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Abstract

Micro-morphological changes precede the appearance of visible damage after herbicide application and are essential in providing data for the safe recommendation in chemical management of weeds. Therefore, the aim of this research was to verify the anatomical changes of leaf tissue caused by application of herbicides in cassava (*Manihot esculenta* Crantz. cv. IAC-12). A greenhouse experiment was conducted with post-emergence herbicides treatments as follows: nicossulfuron (60 g a.i ha⁻¹), fluazifop (250 g a.i ha⁻¹), fomesafen (250 g a.i ha⁻¹), metribuzin (480 g a.i ha⁻¹), oxyfluorfen (720 g a.i ha⁻¹) and the mixture fluazifop + fomesafen (200 + 250 g a.i ha⁻¹), and an untreated control, respectively. The results obtained have allowed to affirm the cassava plants (cultivar IAC-12), exhibited changes in leaf anatomy in response to herbicide application even on cassava leaves without no visual toxicity symptoms. The products caused alterations both in tissue thickness as in tissue proportion in the leaf blade. For the fluazifop, a eudicotyledonous selective herbicide, changes were observed in tissue thickness and proportion of leaf blade, even without any visual toxicity detected. Cassava plants (IAC-12), showed structural changes in leaf anatomy in response to application of herbicides. The leaf anatomy of cassava cv. IAC-12, can be used to indicate the herbicide effect on cassava (*Manihot esculenta* Crantz. cv. IAC-12) plants.

Keywords: Post-emergence herbicides, selectivity, tissue thickness and proportion, visual toxicity, weed chemical management.

Resumo

Mudanças micro-morfológica precede o aparecimento de danos visíveis após a aplicação do herbicida e são essenciais no fornecimento de dados para a recomendação segura no manejo químico de ervas daninhas. Assim, o objetivo deste trabalho visou verificar as alterações anatômicas do tecido foliar causada pela aplicação de herbicidas em mandioca (*Manihot esculenta* Crantz. cv. IAC-12). Um experimento foi conduzido em ambiente protegido com os seguintes herbicidas aplicados em pós-emergência na cultura da mandioca: nicossulfuron (60 g i.a ha⁻¹), fluazifop (250 g i.a ha⁻¹), fomesafen (250 g i.a ha⁻¹), metribuzin (480 g i.a ha⁻¹), oxyfluorfen (720 g i.a ha⁻¹), a mistura fluazifop + fomesafen (200+250 g ha⁻¹), e um tratamento sem aplicação de herbicidas. Os resultados obtidos permitem afirmar que as plantas de mandioca (cultivar IAC-12) apresentaram mudanças na anatomia foliar, em resposta à aplicação do herbicida até mesmo em folhas sem nenhum sintoma visual de intoxicação. Os produtos causaram alterações tanto na espessura do tecido como na proporção de tecidos na lâmina de folha. Para o fluazifop, um herbicida seletivo para espécies eudicotiledôneas foram observadas alterações na espessura do tecido e na proporção de lâminas foliares, mesmo quando não foram detectadas sintoma visual de intoxicação nas folhas. As plantas de mandioca (IAC-12) apresentaram mudanças estruturais na anatomia foliar em resposta à aplicação de herbicidas. A anatomia da folha da mandioca (*Manihot esculenta* Crantz. cv. IAC-12) é um parâmetro para indicar o efeito de herbicidas em relação as avaliações visuais.

Palavras-chave: Espessura e proporção do tecido, intoxicação visual, herbicidas pós-emergência, manejo químico de plantas daninhas. Seletividade.

Introduction

Cassava is a eudicotyledonous, Euphorbiaceae and genus *Manihot* (Carvalho, 2013). The species *Manihot esculenta* Crantz., is a traditional crop in tropical regions, producer of carbohydrates that provides food from their roots to humans (da Silva *et al.*, 2016) and animals (Yuquilema *et al.*, 2014).

Life cycle of cassava can reach up to two years and weed competition can reduce roots production. Besides that, the lack of registered herbicides is a problem and difficult weeds management (Silva *et al.*, 2012; Braga *et al.*, 2014). Also there is lack of studies related to the selectivity of new herbicides, especially for application post-emergence of cassava. This fact makes some growers to use herbicides without registration and non-selective to the crop, damaging its productivity (Silva *et al.*, 2012).

The presence of weeds alter cassava growth and development, affecting its photosynthetic characteristics (Aspiázú *et al.*, 2010), with a consequent size reduction, weight and number of roots. The response of cassava plants to herbicide application varies from overall selectivity to severe impairment of the production due to the toxicity caused to the crop (Silva *et al.*, 2012). Therefore, the basis for the success of the practice in agriculture is the selectivity of the herbicides, which can be defined as the differential response of the plant species to the application of a specific molecule. Thus, the intensity of application and the formulation used are extremely relevant information on crop management, regarding the unwanted contact of the herbicide at the time of application (Tuffi-Santos *et al.*, 2009). Furthermore, effects of herbicides on tissues level may not be seen and, thereby, decrease the ability in cultural competition after a period of effective control by the herbicide (Pavlovic *et al.*, 2013). However, there is an information gap regarding the effect of different molecules in leaf anatomy of cassava plants. Since micromorphological changes precede the appearance of visible damage (Tuffi Santos *et al.*, 2007), demonstrating that microscopic studies provide additional important data.

Metribuzin is a photosystem II inhibitor, acting in the chloroplast by competing with partially reduced plastoquinone “Qb” (QBH) for the site at the D-1 protein, causing an output of plastoquinone and interrupting the flow of electrons between photosystems (Silva & Silva, 2007). Other products such as oxyfluorfen and fomesafen inhibit the protoporphyrinogen IX oxidase (PPO) enzyme, this way paralyzing the synthesis of chlorophyll and causing a series of oxidative reactions. After absorption and translocation of these herbicides to the site of

action, the light is always required for their action. Nicosulfuron is an acetolactate synthase inhibitor (ALS), responsible for the synthesis of aminoacidsvaline, leucine and isoleucine, interrupting, thereby, the synthesis of proteins (Silva & Silva, 2007).

According to Ferreira *et al.* (2002a,b) and Procópio *et al.* (2003), the anatomical study of leaves can improve understanding of the barriers that each species imposes on herbicide penetration and, thus, contribute to the search for strategies that overcome these obstacles. Therefore, leaf anatomical analysis can be successfully applied in the identification of species that are susceptible, tolerant or resistant to a given chemical, as well as in the description of phytotoxic symptoms, contributing to studies of herbicide selectivity to terrestrial or aquatic species.

Therefore, there is a need to assess the injuries caused in the leaf tissue of cassava resulting from the application of herbicides that may harm plant physiological functions. Even if not observing visible effects on the plant canopy, the continued use of such products may, in the medium and long term, compromise the production. Given these concerns, the aim of this research was to verify the anatomic changes on leaf tissue caused by fluazifop, fomesafen, metribuzin, nicosulfuron, oxyfluorfen post-emergence herbicides and the mixture fluazifop + fomesafen herbicides used to control weeds in cassava (*Manihot esculenta* Crantz. cv. IAC-12) plants in Minas Gerais, Brazil.

Materials and methods

Preparation of substrate

The experiment was conducted in a green house, with controlled conditions of temperature and humidity. It was used a Red Yellow Latosol soil with clay texture. Soil physical and chemical analysis showed in Table 1, according to EMBRAPA soil classification.

Table 1. Results of soil physical-chemical analysis

| Soil | pH (H ₂ O) | P (cmol _c dm ⁻³) | K | Ca ²⁺ | Mg ²⁺ | Al ³⁺ | H+Al | (t) |
|----------------|--------------------------|--|----|------------------|------------------|------------------|------|-----|
| LRY | 5.4 | 1.4 | 10 | 0.5 | 0.2 | 0.4 | 4.4 | 1.7 |
| Textural Class | | | | | | | | |
| Clay | | Silt | | | | Sand | | |
| 56% | | 6% | | | | 38% | | |

Analyses carried out according to the methodology of the Brazilian Agricultural Research-EMBRAPA (1997).

To ensure adequate nutrition on the substrate, 300 kg ha⁻¹ of lime (PRNT = 80%), 220 kg ha⁻¹ of single superphosphate (18% P₂O₅) and 40 kg ha⁻¹ of potassium chloride (58% K₂O) was applied. Nitrogen fertilization was carried out in top-dressing 30 days after crop emergence, at an application rate 40 kg ha⁻¹ of previously dissolved in water urea (44% N). Irrigation was done daily by automatic microaspiration system.

Treatments used and experimental design

Treatments consisted of post-emergence application of the following herbicides: nicosulfuron (60 g a.i ha⁻¹), fluazifop (250 g a.i ha⁻¹), fomesafen (250 g a.i ha⁻¹), metribuzin (480 g a.i ha⁻¹), oxyfluorfen (720 g a.i ha⁻¹) and the mixture fluazifop + fomesafen (200 + 250 g a.i ha⁻¹), plus an untreated control. It was used a randomized complete block design, with four repetitions. Each vase with volumetric capacity of 5 L, containing substrate, represented an experimental unit. The cuttings from the cultivar IAC-12 were acquired from cassava farmers and were planted in March 2010, being the sproutings visible five days later.

Herbicide application

Herbicides were applied 60 days after planting, when the plants were about 20 cm high and had about 15 fully expanded leaves. Herbicide application was established using a CO₂ pressurized backpack sprayer, with constant pressure, equipped with a spear containing a type range nozzle tip, working at a height of 50 cm from the target, with a speed of 1 m s⁻¹ and spray volume of 200 L. ha⁻¹.

Assessments

Anatomical assessments of cassava leaves were carried out from to collecting five leaves fully expanded in each repetition and that receive herbicide spraying. The leaves were collected seven days after herbicide application. Posteriorly, they are fixed in a mixture of formaldehyde acetic acid (FAA) and alcohol 70% in the ratio 0.5:0.5:9.0 (FAA 70%), being later transferred to ethanol 70%. The anatomic sections were made free-hand, with the aid of a blade, in the middle region of the leaf in the transverse direction (cross sections) and stained with alcian blue 0.5% in tartaric acid 2 % and fuchsin 0.05 %. All of material was mounted among slide and coverslip with glycerinated gelatin, totalizing 15 blades that it were. The slides were observed and photographed under an optical microscope Olympus BX 60 model coupled to the digital camera Canon A630. The images were analyzed for UTHSCSA ImageTool software image analysis, by measuring five fields

per replicate for each analyzed variable (Ribero *et al.*, 2012).

Evaluations were made of the following characteristics: leaf blade thickness (LB), thickness of the palisade parenchyma (EAD), spongy parenchyma (PL), thickness of the adaxial epidermis (EAD) and thickness of the adaxial wall (PD). Other variables were also calculated from area measurements: proportion of adaxial epidermis (% EAD), proportion of palisade parenchyma (% PP), proportion of spongy parenchyma (% PL) and proportion of abaxial epidermis (% EAB), as well as the visual integrity of the tissues.

The visual intoxication was performed from to comparing the plants subjected to herbicide application with cassava control plants. Symptoms, when present, can be viewed through the pictures. This was carried out just to detect typical symptoms caused by the herbicides used.

Statistic analysis

Data were subjected to analysis of variance. To identify significant difference among treatments and statistical significance for all comparisons was made at p<0.05. Tukey's multiple range test was used to compare the mean values of treatments.

Results and discussion

Visual intoxication

In the visual analysis of the plants under treatments injuries were observed, such as the curling of leaves, in plots treated with metribuzin (Figure 1-F), wilting and beginning of chlorosis in plots which received fomesafen and nicosulfuron (Figure 1-B, 1-C), respectively.

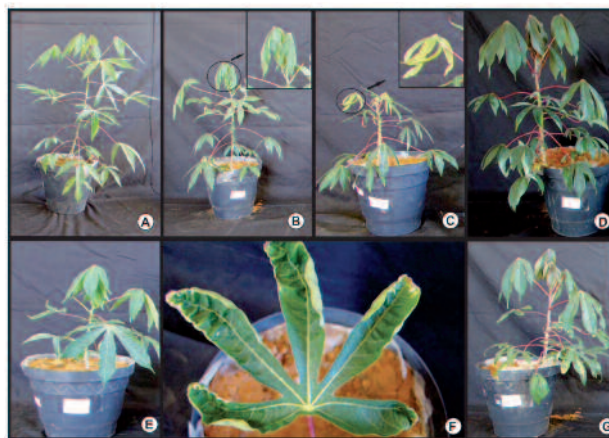


Figure 1. Control (A) and cassava plants treated with herbicides: fomesafen (B), nicosulfuron (C), fluazifop-p-butyl (D), oxyfluorfen (E), metribuzin (F) and fluazifop + fomesafen mixture (G).

The observed chlorosis can be reflexes of the degeneration of chloroplasts and, or, inhibition of chlorophyll formation caused by herbicides, particularly the inhibitors of PPO (protoporphyrinogen oxidase), as is the case of fomesafen (Dayan & Zaccaro, 2012). According to Rodrigues & Almeida (2005), this herbicide is activated by light and destroys the cell membranes, causing necrosis and tissue death.

Chlorosis in older leaves and wilting in young leaves were observed in the plots in which was applied the mixture fluazifop + fomesafen (Figure 1-G), while plants treated with fluazifop did not show apparent visual symptoms (Figure 1-D). Indicating that in the mixture of fluazifop and fomesafen, there are not synergistic effects and the damage to cassava leaves are the caudate fomesafen.

The leaves of cassava plants treated with oxyfluorfen, suffered curling and development interruption (Figure 1-E). Therefore, this provides information of this herbicide is non-selective for cassava plants post-emergence, as well as pre-emergence application (Biffe *et al.*, 2010).

Anatomical analysis

Anatomical analysis of the cassava leaf blade in control plants, revealed that the epidermis presented cells on the adaxial surface with predominantly regular format, with external periclinal walls straight and covered with cuticle, and slightly sinuous anticlinal walls (Figure 2-A). On the abaxial surface, the epidermis showed cells forming papillae distributed all over the leaf. Such conformations have already been described as aids in protecting the plant against excessive water loss and better convergence of diffuse radiation, helping thus, the net photosynthetic rate (Torres *et al.*, 2012). It presents dorsiventral mesophyll with a uniseriate palisade parenchyma. The spongy parenchyma varies in shape and is arranged parallel to the leaf surface, immediately below the palisade parenchyma, as found for this genre by Moraes-Dallaqua & Coral (2002), (Figure 2).

Plants treated with the mixture of herbicides fluazifop + fomesafen showed decrease in thickness (approximately 75%) of the adaxial wall (PD) compared to the control. For all other tested herbicides, no difference was observed in comparison to control (Table 1). Silva *et al.* (2014), assessing physiological and Silva *et al.* (2011), evaluating growth characteristics of five cassava culture where were observed high sensitivity to this herbicide mixture, demonstrating the reduction in thickness of the DP, which can also be used as selective herbicides indicator for cassava plants.

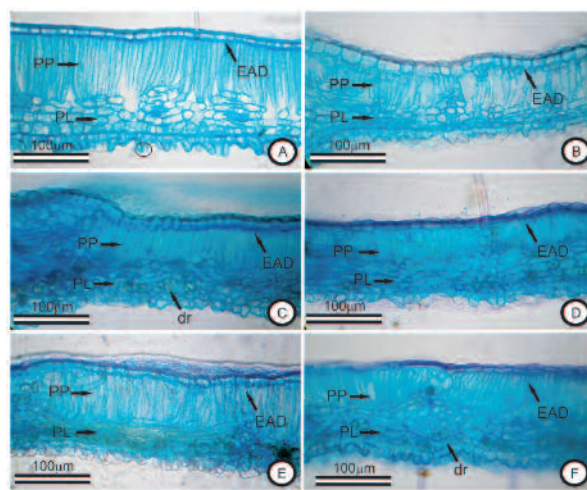


Figure 2. Cross sections of cassava leaves in the middle region, evidencing adaxial epidermis (EAD), palisade parenchyma (PP), spongy parenchyma (PL) of control (A) and herbicide treatments: fluazifop (B), fomesafen (C), fluazifop + fomesafen (D), oxyfluorfen (E) and nicosulfuron (F).

All the tested herbicides significantly affected the thickness of the adaxial epidermis (EAD) in cassava leaves, being nicosulfuron the one which caused the greatest decrease in EAD, corresponding to about 60 % compared to control. The fluazifop, while not presenting visual intoxication symptoms in cassava leaves (Figure 1-D), caused a reduction of approximately 50% in the EAD (Table 1). Costa *et al.* (2011), in an effort to study the effect of herbicides 2,4-D and glyphosate in *Polygonum lapathifolium*, verified that they caused changes in EAD, and the authors found reductions of 50.2 and 42.2 %, respectively, compared to the control, 30 days after treatment application.

In general, the tested herbicides have allowed a decrease in the leaf blade thickness and tissues evaluated (Table 1). When evaluating the effect of herbicides on the proportion of cassava leaf tissue, it was found that, for adaxial epidermis (%EAD), the treatments that received fomesafen, fluazifop and fluazifop + fomesafen did not differ from the control, being metribuzin, the herbicide that affected more the %EAD, leading to a reduction of approximately 35% in this variable (Table 2).

With respect to the proportion of palisade parenchyma (%PL) in cassava leaves, it was observed that the fluazifop, metribuzin, nicosulfuron and oxyfluorfen herbicides, promoted increments of 15, 30, 3 and 27%, respectively, in the proportion of this tissue, compared to the treatment without herbicides. However, fomesafen and the mixture fluazifop + fomesafen have allowed a reduction in the %PP (Table 2).

Table 2. Thickness of adaxial wall (PD), thickness of adaxial epidermis (EAD), thickness of palisade parenchyma (PP), thickness of spongy parenchyma (PL) and thickness of leaf blade (LB) of cassava leaves treated with fluazifop, fomesafen, metribuzin, nicosulfuron, oxyfluorfen and fluazifop + fomesafen herbicides.

| Treatments | PD | EAD | PP | PL | LB |
|-----------------------|--------------|-----------|----------|---------|----------|
| | -----µm----- | | | | |
| Control | 5,73 a | 20,15 a | 95,26 a | 97,53 a | 218,68 a |
| Fluazifop | 4,71 a | 10,08 cd | 51,42 bc | 58,47 b | 127,83 c |
| Fomesafen | 5,25 a | 11,87 bcd | 53,12 bc | 57,81 b | 133,08 c |
| Metribuzin | 6,24 a | 12,77 bcd | 47,36 bc | 69,45 b | 135,83 c |
| Nicosulfuron | 4,08 a | 9,37 d | 97,30 a | 66,26 b | 166,48 b |
| Oxyfluorfen | 6,49 a | 12,88 dc | 42,25 c | 69,61 b | 131,25 c |
| Fluazifop + Fomesafen | 1,42 b | 14,70 b | 69,29 b | 93,37 a | 187,79 b |
| CV (%) | 22,44 | 11,27 | 14,47 | 9,35 | 6,20 |

*Means followed by the same letter in the column do not differ significantly at the 0-05 level by the tukey Test.

When assessing the proportion of spongy parenchyma (%PL), it was found that the fomesafen, nicosulfuron and fluazifop + fomesafen herbicides, promoted increases of about 9, 4 and 13%, respectively, in the %PL. The other herbicides, led to a decrease in the values of this variable compared to the control, corresponding to 15, 15 and 22% for fluazifop, metribuzin and oxyfluorfen herbicides, respectively (Table 2).

The proportion of abaxial epidermis was not affected by herbicide application (Table 2). Therefore, in general, all tested herbicides have caused changes in the proportion of leaf blade tissue in cassava plants, considering a situation of absence of stress caused by herbicides, the proportion of tissues, on average, was approximately 10, 40, 38 and 10% for adaxial epidermis, palisade parenchyma, spongy parenchyma and abaxial epidermis, respectively (Table 2). The fluazifop, considered a selective herbicide for cassava (Silva *et al.*, 2012; Silva *et al.*, 2011) even without causing visual symptoms of toxicity in the leaves of the cassava plants, caused a change in the proportion of leaf tissues. In this case, it was found an increase in the %PP and a decrease in the %PL (Table 2). On the other hand, metribuzin and oxyfluorfen have allowed an increase in the %PP, comparing to the control, of about 50%, and reductions in %PL to 30% (Table 3).

Table 3. Evaluation of tissue proportions: adaxial epidermis (%EAD), palisade parenchyma (%PP), spongy parenchyma (%PL) and abaxial epidermis (%EAB) of the leaf blade of cassava plants submitted to fluazifop-p -butyl, fomesafen, metribuzin, nicosulfuron, oxyfluorfen and the mixture fluazifop + fomesafen herbicides

| Herbicide | %EAD | %PP | %PL | %EAB |
|-----------------------|---------|----------|-----------|---------|
| Control | 10,37 a | 40,55 bc | 38,92 abc | 10,15 a |
| Fluazifop | 9,77 ab | 46,47ab | 33,84 bc | 9,90 a |
| Fomesafen | 10,33 a | 35,94 c | 42,51 a | 11,20 a |
| Metribuzin | 6,79 c | 51,7 a | 33,73 bc | 7,74 a |
| Nicosulfuron | 8,24 bc | 41,33 bc | 40,66 bc | 9,75 a |
| Oxyfluorfen | 8,82 c | 51,43 a | 30,42 c | 9,31 a |
| Fluazifop + Fomesafen | 10,64 a | 35,38 c | 44,09 a | 9,87 a |
| CV (%) | 9,08 | 7,80 | 9,68 | 9,40 |

* Means followed by the same letter in the column do not differ significantly at the 0-05 level by the tukey Test.

Conclusion

Cassava (*Manihot esculenta* Crantz. cv. IAC-12) plants, showed structural changes in leaf anatomy in response to application of metribuzin, fomesafen, nicosulfuron, fluazifop, oxyfluorfen herbicides and the mixture of fluazifop + fomesafen post-emergence herbicides, even without visible leaf injuries. The cassava leaf anatomy can be used to indicate the herbicide effect on cassava.

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